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Oridonin induces apoptosis in uveal melanoma cells by upregulation of Bim and downregulation of Fatty Acid Synthase

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ABSTRACT

Oridonin is an orally available drug isolated from Traditional Chinese Medicine. Previous studies with oridonin have demonstrated broad-spectrum anticancer activity in a variety of cancer types. However, the effect of oridonin in uveal melanoma has not been addressed. In this study, we aimed to investigate whether oridonin elicited anticancer activity and its underlying mechanism in human uveal melanoma cells. We demonstrated that oridonin potently reduced cell viability, induced apoptosis and inhibited clonogenic survival and growth with single digit micromolar concentrations in uveal melanoma OCM-1 and MUM2B cell lines. We found that oridonin markedly increased the expression of proapoptotic Bcl-2 family protein Bim in uveal melanoma cells, and knockdown Bim by small interfering RNA significantly attenuated oridonin-induced cell death, indicating an essential role of Bim in oridonin-mediated anticancer activity. Additionally, we observed that oridonin suppressed Fatty Acid Synthase (FAS) expression in uveal melanoma cells, and enforced FAS expression by insulin partially rescued the cells from oridonin-induced apoptosis, showing that inhibition of FAS also contributed to oridonin-mediated apoptosis. Taken together, we reported that oridonin displays potent anticancer effect against uveal melanoma cells through upregulation of Bim and inhibition of FAS. Since oridonin is a popular anticancer agent, our study therefore may have translational implication on the management of patients with uveal melanoma.

1. Introduction

Uveal melanoma is the most common primary intraocular tumors in adults. Patients with localized disease can be cured with enucleation, and often have a good prognosis. However, up to 50% of patients with uveal melanoma will develop liver metastases within an average time of 2.4 years [1,2]. Moreover, once metastasized, uveal melanoma becomes more resistant to systemic chemotherapy and radiology, and the average survival for patients is only from 2 to 14 months. Therefore, novel therapeutic strategies need to be explored for improving the survival of patients with uveal melanoma [1,2].

Traditional Chinese Medicine (TCM) has been widely and successfully used in treating human cancer [3,4]. Isodon plant *Rabdosia rubescens*, a Chinese herb is used broadly in our Henan province to suppress disease progress, reduce tumor burden, alleviate syndrome and prolong survival for patients with esophageal and stomach tumors, and many other medical conditions since ancient time [3]. Oridonin is a natural diterpenoid purified from *R. rubescens*. Recent studies found that oridonin has remarkable anticancer activities in a variety of cancers types, including esophageal cancer, breast cancer, hepatocellular cancer, colorectal cancer, and pancreatic cancer, while sparing normal human cells such as lymphoid cells and fibroblasts [4–7]. Notably, several studies reported that this drug also exhibits significant anticancer activity in skin melanoma. For instance, Zhang et al. reported that oridonin induced cell killing in A375 melanoma cell line [8]. Satooka et al. reported that oridonin showed strong cytotoxicity against murine B16-F10 melanoma [9]. These evidences demonstrate that oridonin has broad-spectrum anticancer activity. However, whether oridonin exerts anticancer effects in uveal melanoma has not been investigated.

In this study, we investigated anticancer potential and underlying molecular mechanism of oridonin in uveal melanoma. We

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demonstrated that oridonin potently impairs the capability of survival and proliferation of uveal melanoma cells by induction of apoptosis. Our findings indicated that oridonin might be a treatment option for patients with uveal melanoma.

2. Materials and methods

2.1. Cell lines and compound preparations

Human primary, invasive and metastatic potential uveal melanoma cell lines OCM-1 and MUM2B purchased from China Center for Type Culture Collection (Wuhan, China) were maintained in Dulbecco Modified Eagle Medium (DMEM) (HyClone/Thermo Fisher Scientific, Beijing, China) supplemented with 10% heat-inactivated fetal FBS (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd., Hangzhou, China) in a humidified atmosphere of 5% of CO_2 in air at 37 °C. Oridonin was purchased from Shanxi Huike Plants Exploitation (Xian, China), and the purity was more than 98% as determined by high-performance liquid, and was dissolved in Dimethyl sulfoxide (DMSO) with a stock concentration of 1 mM and stored at −20 °C. Human recombinant insulin was purchased from Life Technologies (Shanghai, China).

2.2. MTT viability assay

Cell viability was evaluated by a colorimetric assay based on 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Briefly, cells were seeded in 96-well plates. After incubation overnight, cells were treated with various concentrations of oridonin. At the end of treatment, MTT dye was added to each well and plates were incubated at 37 °C for 4 h. The supernatants were decanted. Insoluble formazan complexes were solubilized with DMSO and the absorbance was measured at 540 nm using a Benchmark Plus microplate spectrophotometer (Bio-Rad, Hercules, CA). Cell viability inhibition was evaluated as the ratio of the absorbance of the samples to that of the control. Each experimental condition was done in triplicate and performed at least twice.

2.3. Colony forming assay

Colony forming assay was performed as described previously [10]. OCM-1 and MUM2B cell lines were plated into a 6-well culture plate (500 cells/well) and allowed to adhere for 10 h before treatment. After adherence, cells were treated with oridonin at 2.5, 5, and 10 μmol/L. After 48 h, the oridonin-containing medium was removed, and the cells were allowed to form colonies in complete medium for 14 days. Then, the colonies were fixed with a solution of acetic acid and methanol (1:3) for 15 min, stained with 5% Giemsa (Sigma–Aldrich) for 30 min, and counted manually.

2.4. Cell death and flow cytometry apoptosis assays

Cell death induction was quantitated by microscopic examination in a trypan blue exclusion assay. Apoptosis analysis was done using an Annexin V/propidium iodide (PI) apoptosis detection kit (Roche, Shanghai, China) by flow cytometry according to the manufacturer's instructions. Cells positively stained with Annexin V were counted as apoptotic cells.

2.5. Western blotting analysis

Western blotting analyses were performed as described previously [11]. Antibodies used were as followings: anti-PARP (9542), anti-caspase-9 rabbit polyclonal antibody (9502), anti-caspase-3 rabbit polyclonal antibody (9662), anti-Bcl-xl rabbit polyclonal antibody (2762), anti-Mcl-1 rabbit polyclonal antibody (4572), and anti-Bfl-1/A1 (14093), anti-Bim (2819), anti-Bad (9292), anti-Bid (2002), anti-Bax (2772), anti-Bak (3814), anti-FAS (3180) from Cell Signaling Technology (Shanghai, China); anti-Noxa (22764), anti-SREBP-1 (13551) and HRP-conjugated secondary anti-mouse, anti-goat and anti-rabbit antibodies from Santa Cruz Biotechnology Shanghai Co. Ltd. (Shanghai, China).

2.6. RNA interference

RNA interference was performed as described previously [11]. The siRNA transfections were carried out using Lipofectamine RNAiMax transfection reagent (Invitrogen) according to the manufacturer's instructions. Two Bim (BCL2-like 11) siRNA oligos were purchased from Dharmacon (Shanghai, China). FAS siRNA (sc-43758) was purchased from Santa Cruz Biotechnology Shanghai Co. Ltd. A nonspecific nonsilencing siRNA (siCTL) was used as control. Concentration for transfection was 10 nmol/L of each siRNA.

2.7. Statistical analysis

Statistical analyses were performed by one-way ANOVA using SPSS (version 13.0, SPSS Inc, Illinois, USA). $p < 0.05$ was considered statistically significant, $p < 0.01$ was considered very statistically significant.

3. Results

3.1. Oridonin inhibits the viability of uveal melanoma cells

To investigate the inhibitory effect of oridonin on cell viability of uveal melanoma cells, we treated OCM-1 and MUM2B cell lines with various concentrations of oridonin and detected cell viability by MTT assays. We found that oridonin inhibited the viability of OCM-1 and MUM2B cell line in time- and dose-dependent manners (Fig. 1A). Oridonin achieved IC50 values of 3.1 and 1.0 μM in OCM-1 cell line after treatment for 1 day and 3 days, respectively. Oridonin achieved IC50 values of 6.1 and 2.2 μM in MUM2B cell line after treatment for 1 day and 3 days, respectively. Notably, oridonin at 4–10 μmol/L completely inhibited cell viability of the two cell lines. These results demonstrated that oridonin has potent inhibitory ability against uveal melanoma cells.

3.2. Oridonin inhibits colony formation of uveal melanoma cells

We next determined whether oridonin had long-term anticancer activity in uveal melanoma cells using the flat plate colony forming assay (Fig. 1B). We found that both cell lines formed decent number of clones when they were treated with DMSO control. By contrast, oridonin dose-dependently inhibited colony formation. Oridonin at 10 μM abrogated the ability to form clones (data not shown), and at 2.5 and 5 μM significantly reduced the clone numbers in both cell lines as compared to DMSO treatment (Fig. 1B). These results demonstrated that oridonin potently impaired the capability of long-term survival and proliferation in uveal melanoma cells.

3.3. Oridonin induces apoptosis of uveal melanoma cells

Since previous studies have shown that oridonin induced apoptosis in breast cancer, colon cancer and several types of hematological cancers [5–8], and also since we noticed that treatment with oridonin for 24 h resulted in most cells showing characteristic apoptosis morphological changes such as shrinkage, rounding, and floating in both cell lines (data not shown), we investigated

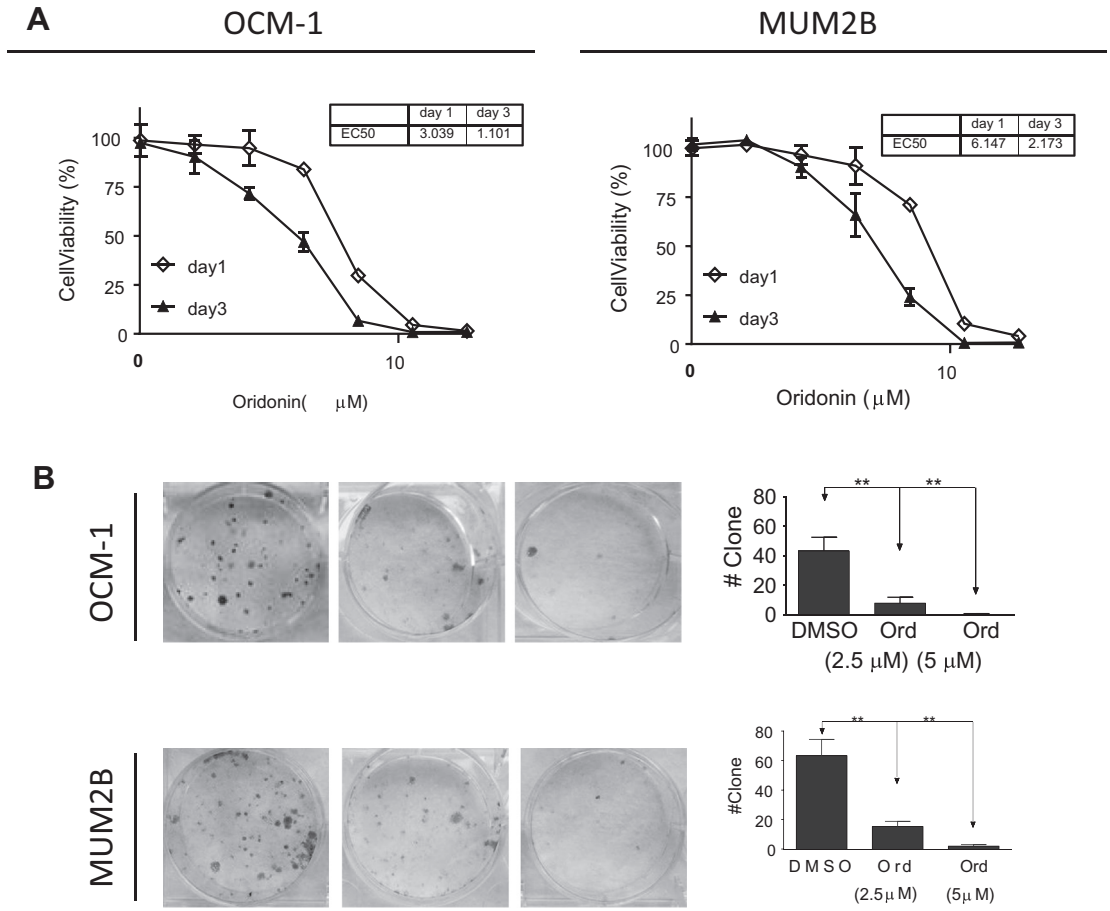

Fig. 1. Oridonin displays potent anticancer activity in uveal melanoma cells. (A) OCM-1 and MUM2B cell lines were treated with different concentrations of oridonin for 24 and 72 h, cell viability inhibition was determined using an MTT assay. (B) OCM-1 and MUM2B cell lines were seeded into six-well plates at 500 cells per well in triplicates, and treated with oridonin for 2 weeks, followed by 0.05% methylene blue staining and colony counting. (left panels) Representative results show photographs of stained 6-well plates for OCM-1 and MUM2B, respectively. (right panels), Data show means ± S.D.

whether this drug had apoptotic effect in uveal melanoma cells. We treated OCM-1 and MUM2B cell lines with oridonin at 5 μM for 48 h and stained with annexin V-FITC and PI for flow cytometric analysis. We found that oridonin treatment induced a significant increase in both early (Annexin-V positive/PI negative) and late stage (Annexin-V positive/PI positive) apoptosis in both OCM-1 and MUM2B cell lines (Fig. 2A and B) as compared to DMSO control treatment. Oridonin at 5 μM induced apoptosis in 56% OCM-1 cells, an increase of 47% when compared to the number of apoptotic cells in the control group (Fig. 2B, left panel). Oridonin at 5 μM induced apoptosis in 37% OCM-1 cells, an increase of 28% when compared to the number of apoptotic cells in the control group (Fig. 2B, right panel).

Apoptosis is carried out by the cascade of caspase activation [12]. To further investigate the apoptotic pathway triggered by oridonin, we investigated the activation of several apoptosis biomarkers with western blotting analysis. We noted that treatment with oridonin for 24 h triggered cleavage of initiator procaspase-9, activation of effector caspase-3 and cleavage of PARP (Fig. 3A). To determine the role of caspases activity in oridonin-mediated apoptosis, we pretreated the cells with pancaspase inhibitor z-VAD-fmk, then followed by treatment with oridonin and examined the cell death induction. We found that pretreatment with pancaspase inhibitor significantly reduced the cell death induction by 28% and 33% in OCM-1 and MUM2B cell lines, respectively (Fig. 3B). Collectively, these results demonstrated that caspases-dependent apoptosis induction contributes essentially to oridonin-mediated anticancer activity in human uveal melanoma cells.

3.4. Upregulation of Bim contributes essentially to oridonin-mediated anticancer activity in uveal melanoma cells

Previous studies have shown Bcl-2 family proteins involve in oridonin-induced apoptosis in other kinds of cancers, we next analyzed alteration of the expression of a panel of 9 critical Bcl-2 family proteins in OCM-1 and MUM2B cell lines after treatment with oridonin. Western blotting analysis showed that oridonin treatment had little or modest effect on the expression levels of Bcl-xl, Mcl-1 and Bfl-1/A1, three major antiapoptotic Bcl-2 family members, as well as on the level of the antiapoptotic proteins Noxa, Bad, Bid, Bax, Bak in uveal melanoma cell lines (Fig. 4A and Supplementary Fig. 1A). In contrast, oridonin treatment considerably increased the expressions of all three forms of the proapoptotic protein Bim, including BimEL (extra-long form), BimL (long form) and BimS (short form).

In an attempt to understand the role of Bim in oridonin-mediated anticancer activity, we employed two Bim siRNA oligos. Western blotting analysis showed that both two siRNA oligos were able to effectively reduce the expression of Bim in two cell lines as compared to non-target oligo (siCTL) (Fig. 4B and Supplementary Fig. 1B). In cell death induction assay, we observed that cells transfected with Bim siRNA became resistance to oridonin as compared to cells transfected with siCTL in the uveal melanoma cells. Notably, siRNA oligo2, the more efficient siRNA, conferred stronger resistance to oridonin-mediated cell death than oligo1 in both cell lines (Fig. 4B and Supplementary Fig. 1B), providing additional evidence for the important role of Bim upregulation in mediating

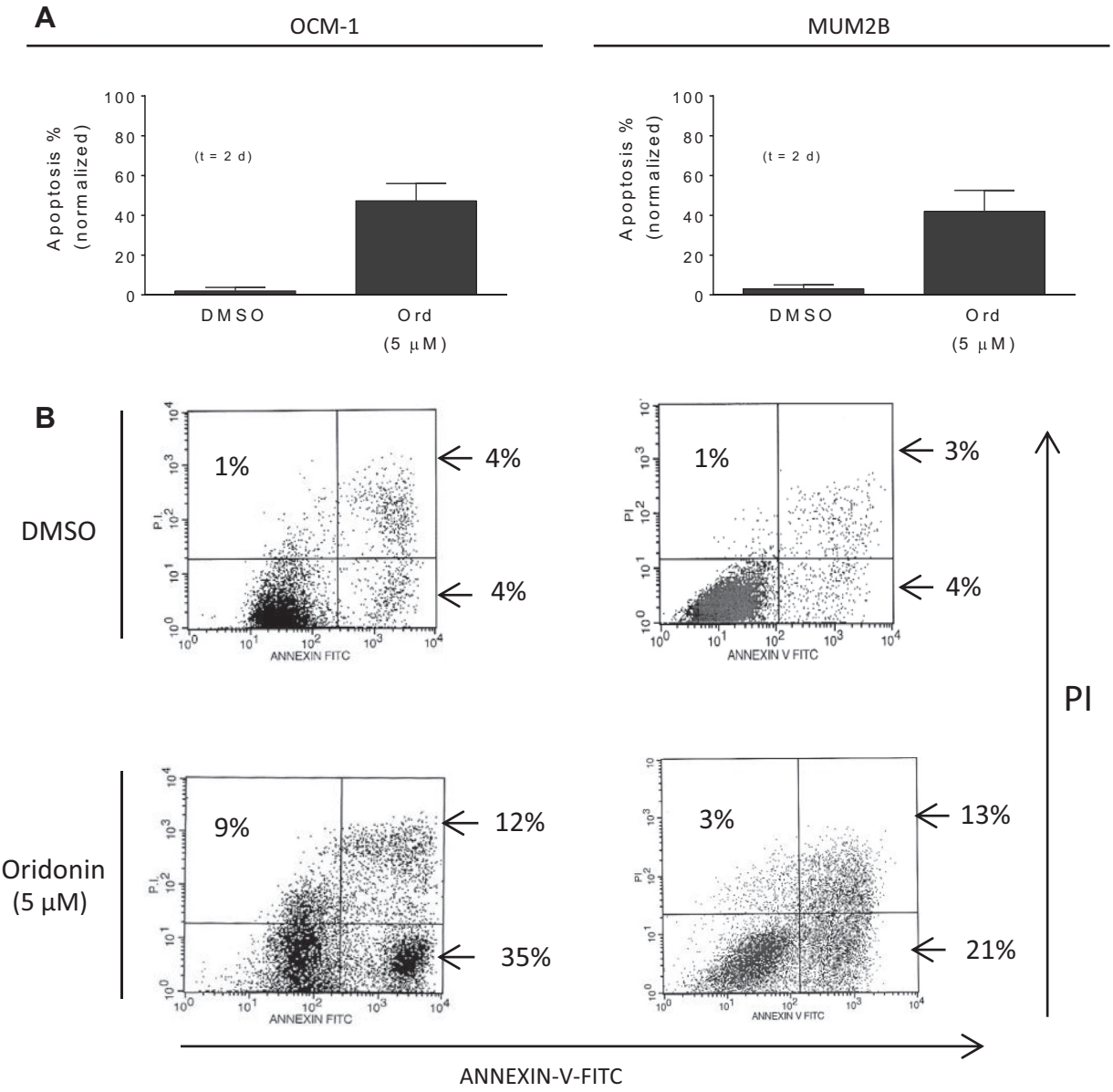

Fig. 2. Oridonin induces apoptosis in OCM-1 and MUM2B. OCM-1 and MUM2B cell lines were treated with oridonin at 5 μM for 48 h, cells were examined with Annexin V/PI staining and examined by flow cytometry assay. (A) Data of Annexin V-positive cells show means ± S.D. of from three experiments. $^{**}p < 0.01$. (lower panel). (B) Representative dot plot of apoptosis assay for CM-1 and MUM2B cell lines, respectively.

anticancer activity by oridonin. Collectively, these results suggested that upregulation of Bim contributed essentially to the anticancer activity of oridonin in uveal melanoma.

3.5. Downregulation of Fatty Acid Synthase (FAS) plays a role in oridonin-mediated anticancer activity in uveal melanoma cells

FAS is the key lipogenic enzyme catalyzing the synthesis of fatty acid. Elevated FAS expression was found in many cancers, and allows those cancer cells to synthesize sufficient fatty acid to support their fast proliferation [12]. FAS has been considered to represent a promising anticancer drug target. Interestingly, a previous study reported that oridonin induced apoptosis by inhibiting FAS through suppressing SREBP1, a transcriptional factor that binds on and activates the FAS gene promoter in colon cancer [12]. We next investigated if oridonin had same effect in uveal melanoma cells. Western blotting analysis showed that treatment with 5 μM of oridonin for 24 h markedly reduced the expression of FAS and SREBP1 in both cell lines, suggesting that oridonin have inhibitory effect on FAS expression in uveal melanoma cells.

We next asked whether the inhibition of these two proteins was required in oridonin-induced apoptosis in uveal melanoma cells using insulin, which has been shown to be able to increase FAS expression through increased AKT and SREBP1 signaling in colon cancer cells [12,13]. Indeed, we found that addition of insulin markedly increased the expression of both SREBP1 and FAS proteins in two uveal melanoma cell lines (Fig. 4C and Supplementary Fig. 1C). Furthermore, cotreatment with insulin significantly (although modestly) inhibited oridonin-mediated cell killing and PARP cleavage, particularly in OCM-1 cell line. These results suggested inhibition of FAS played a partial, but crucial role in oridonin-induced apoptosis in uveal melanoma cells.

To further validate the role of downregulation of FAS in oridonin-induced anticancer activity in uveal melanoma cells, we knocked down FAS by siRNA and examined PARP cleavage and cell death induction in OCM-1 cell line. Western blot analysis showed FAS siRNA transfection efficiently inhibited the expression of FAS

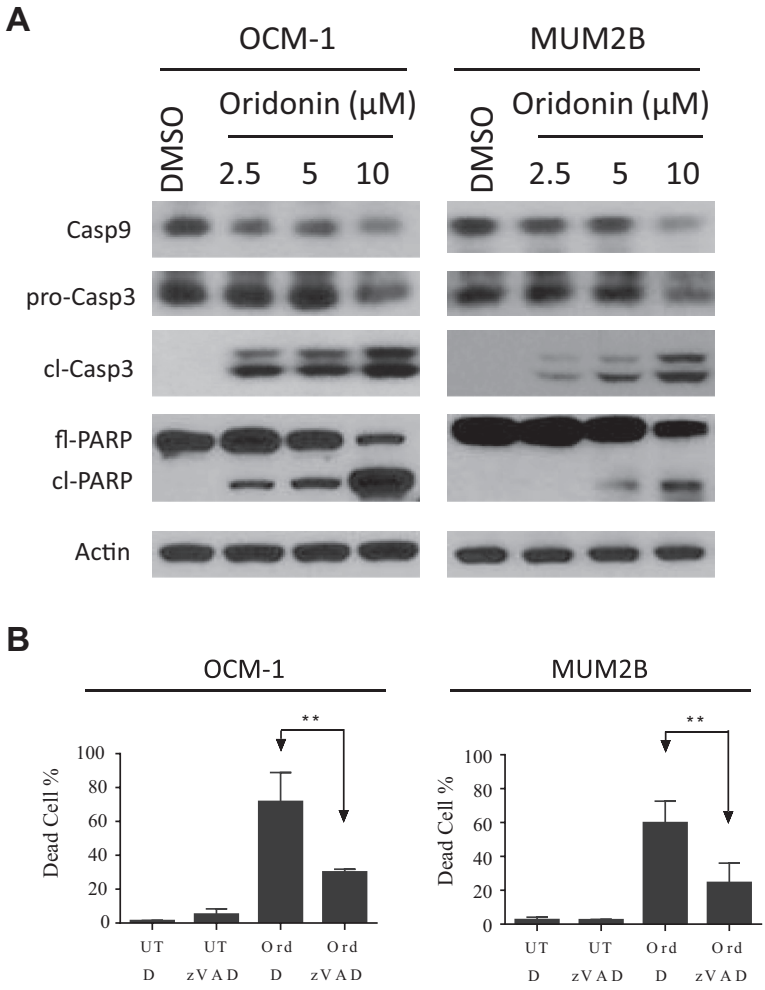

Fig. 3. Oridonin triggers caspase-dependent apoptosis. (A) OCM-1 and MUM2B cell lines were treated with oridonin at 2.5, 5 and 10 μM for 24 h, cells were harvested and lysed. The expression of caspases-9, caspase-3, cleaved caspase-3, as well as PARP was examined by western blotting and specific antibodies. Actin was used as control. (B) OCM-1 and MUM2B cell lines were pretreated with 50 μM of pancaspase inhibitor (Z-VAD.fmk) for 1 h before the treatment with oridonin (5 μM) for 48 h, cell death induction was examined with a trypan blue exclusion assay. $^{**}p < 0.01$.

in the cell line (Fig. 4D). Of note, FAS knockdown triggered cleavage of full-length PARP, and also resulted in 24% of the cell undergoing cell death. These data showed that FAS has a prosurvival role in uveal melanoma cells, and also suggested that oridonin induced apoptosis by FAS inhibition.

4. Discussion

In this study, we have investigated the anticancer activity of oridonin in uveal melanoma cells. We found that oridonin exhibits strong anticancer activity both in primary uveal melanoma OCM-1 cell line, and in metastatic invasive uveal melanoma MUM2B cell line. Importantly, we found that oridonin achieves potent anticancer effect at clinical achievable single digit micromolar concentrations [14]. Taken together, our study suggests possible beneficial effects for patients with uveal melanoma from treatment with oridonin.

The present study indicates that the anticancer activity of oridonin in uveal melanoma cells was mediated chiefly through apoptosis induction. We reached this conclusion by multiple lines of evidences. Firstly, we observed that treatment with oridonin rapidly resulted in most uveal melanoma cells showing characteristic morphological changes associated with apoptosis. Secondly, flow cytometry assays showed that oridonin treatment induced uveal melanoma cells positively stained with Annexin-V-FITC, a direct evidence of apoptosis induction. Thirdly, western blotting analysis showed that oridonin treatment resulted in cleavage of PARP, a key biomarker of apoptosis execution.

Apoptosis signaling pathway is strictly regulated by a fine balance between pro- and anti-apoptotic proteins Bcl-2 family proteins [15,16]. Overexpression of anti-apoptotic Bcl-2 family proteins, such as Bcl-2, Bcl-xl and Mcl-1 has been demonstrated as a major contributing factor for apoptosis resistance in uveal melanoma and many other cancer types [15,16]. Previous studies linked oridonin-mediated anticancer activity to suppression of antiapoptotic Bcl-2 family proteins. For instance, Ikezoe et al. reported that oridonin inhibits Mcl-1 in breast cancer MDA-MB-231 cells during apoptosis induction [17]. Zhang et al. found that oridonin markedly inhibits Bcl-2 in cervical cancer Hela cell line [18]. In this study, we systematically investigated the effect of oridonin on a panel of 9 critical Bcl-2 family proteins in uveal melanoma cells. Nevertheless, we found that treatment with oridonin at a subtoxic concentration had no or minimal effect on the expression of most Bcl-2 family proteins, including the expression of Bcl-xl, Mcl-1 and Bfl-1/A1, three anti-apoptotic Bcl-2 family members commonly overexpressed by uveal melanoma.

Interestingly, a recent study reported that oridonin induces upregulation of BimS in leukemia K562 and HL-60 cells [19]. Since Bim is a potent pro-apoptotic Bcl-2 protein with capability to

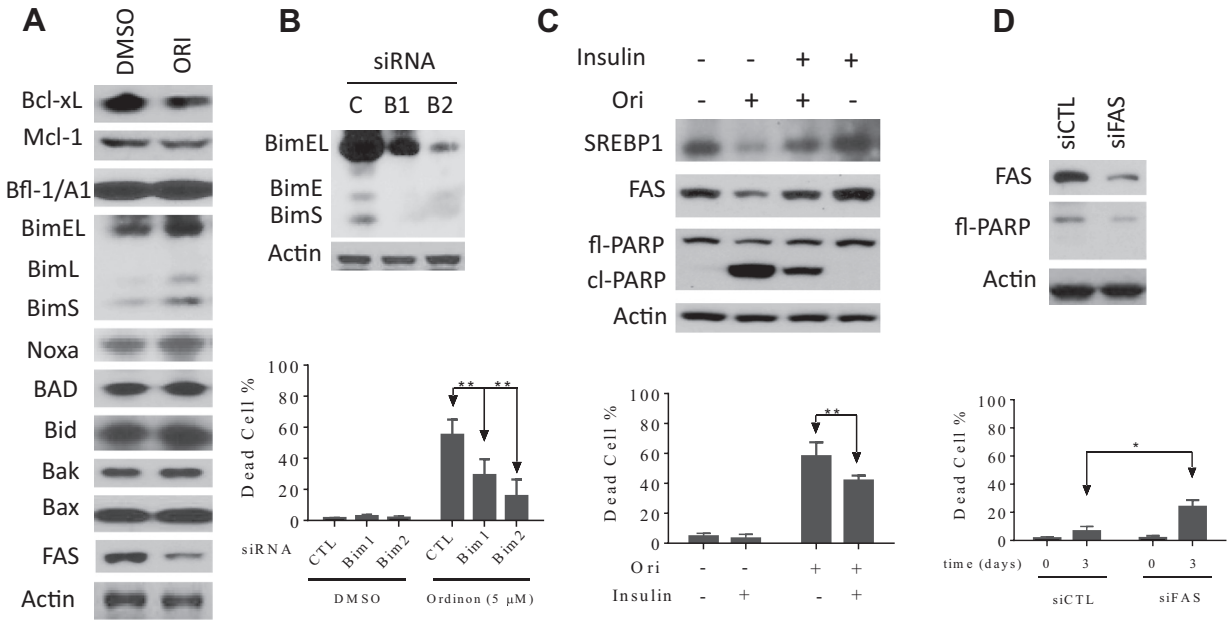

Fig. 4. Oridonin mediates anticancer activity in uveal melanoma cells through upregulation of Bim and suppression of FAS. (A) OCM-1 cell line was treated with 5 μM of Oridonin (Ori) or DMSO for 24 h, cells were harvested and cell lysates were examined by western blotting assays for expressions of Bcl-xl, Mcl-1, Bfl-1/A1, Bim, Noxa, Bad, Bid, Bak, Bax and Fas. Actin was used as a loading control. (B) OCM-1 cell line was transfected with two siRNA oligos against Bim (B1 and B2) for 24 h. (top panel) cells were harvested and siRNA transfection efficiency was examined with western blotting analysis. (lower panel) Transfected cells were treated with oridonin at 5 μM for another 48 h, cell death was examined with trypan blue exclusion assays. (C) OCM-1 cell line treated with insulin at 100 nM alone, Oridonin at 5 μM alone, or both for 24 h. (top panel) Cells were harvested and the expression of SREBP1, Fas, Bim, Mcl-1 and PARP was examined with western blotting analysis. (lower panel) Cells were treated for 48 h, cell death was examined with trypan blue exclusion assays. (D) OCM-1 cell line transfected with siFAS siRNA for 24 h. (top panel) cells were harvested and expressions of FAS and PARP were examined with western blotting analysis. (lower panel) Transfected cells were treated with oridonin at 5 μM for another 48 h, cell death was examined with trypan blue exclusion assays. $^{*}p < 0.05$; $^{**}p < 0.01$.

simultaneously bind and antagonize multiple anti-apoptotic Bcl-2 family members [17,18], we therefore played close attention to role of Bim in the oridonin-mediated anticancer effect in uveal melanoma cells. We observed that oridonin markedly increases the expressions of all three forms of Bim protein in both two uveal melanoma cell lines. Moreover, inhibition of Bim by siRNA approach significantly attenuates the cell killing effect of oridonin in uveal melanoma cells. Collectively, these evidences suggest an essential role of Bim upregulation in oridonin-mediated apoptosis.

Of note, we observed that a pharmaceutical pancaspase inhibitor only partially, but not completely inhibited oridonin-triggered cell death induction. This observation suggests that oridonin might possess other mechanisms in triggering apoptosis in uveal melanoma cells. Given the importance of FAS in blocking caspase-independent apoptosis pathways, such as apoptosis mediated by endoplasmic reticulum stress, generation of reactive oxygen species, and ceramide accumulation, and also that oridonin is capable of suppression of FAS in colon cancer [12], we further investigated the role of FAS in oridonin-mediated anticancer activity in uveal melanoma cells. Our data that inhibition of FAS expression by oridonin or siRNA led to apoptosis, whereas enforced FAS expression conferred resistance to oridonin in uveal melanoma cells, indicate that caspase-independent anticancer activity by oridonin might be triggered by downregulation of FAS.

Overall, our data and previous studies imply that oridonin exhibits anticancer activity through diverse mechanisms depending on cell type. For instance, inhibition of nuclear factor-KB in advanced-stage ovarian cancer cells, interruption of cell cycle progression in breast cancer, induction of endoplasmic reticulum stress in liver cancer, senescence in colorectal cancer cells and autophagy in cervical cancer all have been reported to be involved in oridonin-mediated anticancer activity [20]. These seemingly conflicting evidences highlight the necessary of identifying molecular target(s) of oridonin that is responsible for anticancer activity so as to apply this drug in mechanism-based strategies.

Competing interests

The authors declare that they have no competing interests.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.12.086.

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